



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61L 25/00	A2	(11) International Publication Number: WO 98/57678 (43) International Publication Date: 23 December 1998 (23.12.98)
--	-----------	---

(21) International Application Number: PCT/US98/12687

(22) International Filing Date: 17 June 1998 (17.06.98)

(30) Priority Data:

08/878,471	18 June 1997 (18.06.97)	US
09/099,126	17 June 1998 (17.06.98)	US

(71) Applicant (for all designated States except US): COHESION TECHNOLOGIES, INC. [US/US]; 2500 Faber Place, Palo Alto, CA 94303 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): PRIOR, Jeffrey, J. [US/US]; 602 Spar Drive, Redwood City, CA 94065 (US). SIERRA, David, H. [US/US]; 48 Middle Gate, Atherton, CA 94027 (US). WALLACE, Donald, G. [US/US]; 290 Ringwood Avenue, Menlo Park, CA 94025 (US). DELUSTRO, Frank, A. [US/US]; 2517 DeKoven Avenue, Belmont, CA 94002 (US).

(74) Agents: AXFORD, Laurie, A. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: COMPOSITIONS CONTAINING THROMBIN AND MICROFIBRILLAR COLLAGEN, AND METHODS FOR PREPARATION AND USE THEREOF

(57) Abstract

The present invention relates to thrombin-containing hemostatic compositions, their preparation and use. In particular, it relates to hemostatic compositions comprising stabilized thrombin and microfibrillar collagen in an aqueous medium. In a preferred embodiment of the present invention, the compositions are used in a kit comprising two different components, one of which is autologous patient's plasma as the source of fibrinogen, and the other of which is the thrombin-containing composition which also contains microfibrillar collagen.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

**COMPOSITIONS CONTAINING THROMBIN AND MICROFIBRILLAR
COLLAGEN, AND METHODS FOR
PREPARATION AND USE THEREOF**

5

10

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No. 08/878,471 filed June 18, 1997, the specification of which is incorporated herein in its entirety.

15

TECHNICAL FIELD

The present invention relates to compositions for use in the field of tissue treatment and repair. More particularly, the subject matter of the present invention relates to hemostatic compositions comprising thrombin and microfibrillar collagen.

20

BACKGROUND ART

25

The physiological pathway which is involved in hemostasis and tissue repair is initiated by the release of thromboplastin from injured cells. Upon contact with factor VII in the surrounding plasma, factor X activator is formed. Together with factor V, along with associated phospholipids and calcium, prothrombin is converted into thrombin. The enzymatic activity of thrombin results in cleavage of fibrinogen to form fibrin monomers, which aggregate and are covalently crosslinked via the activity of factor XIIIa (which is formed via thrombin activation of factor XIII). See Figure 1.

30

In order to promote rapid hemostasis and wound healing, many clinicians have focused on the development of hemostatic compositions which include various coagulation factors and function by exploiting the body's own hemostatic processes. For example, fibrin powder has been used as a hemostatic agent for many years. In the mid 1900s,

surgeons began to use fibrin-containing preparations as *in situ* fibrin polymerizing agents. To initiate fibrin clot formation in such compositions, concentrated plasma and ground tissue (muscle or lung) were often used as the catalyst for peripheral nerve anastomosis (Young, et al., *Lancet* 2:126-8, 1940.) In 1944, Tidrick *et al.* utilized human plasma and bovine thrombin in solution as a two-part sealant to attach skin grafts (Tidrick, et al., *Surgery* 15:90-95 (1944)). The use of plasma and thrombin for these and other applications soon fell out of favor due to premature failure of the bond. This was attributed to low mechanical strength, which was in turn attributed to low fibrinogen concentration.

Fibrin sealants utilizing concentrated fibrinogen in the form of homologous cryoprecipitate were reported by Matras, et al. in *Oral Maxillofac. Surg.* 43:605-611 (1985). These types of fibrin sealants are commercially available under the name Tisseel® (Immuno AG, Vienna, Austria) and Beriplast P® (Centeon AG, Germany), and are reviewed in Sierra, *J. Biomater. Appl.*, 7:309-352 (1993). However, these types of fibrin sealants generally involve the administration of pooled blood products.

In an effort to avoid the problems associated with administration of pooled blood products, some investigators turned to the use of tissue sealant compositions utilizing patient autologous plasma as a source of fibrinogen, which was then applied in combination with bovine topical thrombin in calcium chloride solution (*see, e.g.,* Siedentop, et al., *Laryngoscope* 95: 1074-1076 (1985)). However, performance of these materials was somewhat limited because of their inferiority in comparison to the ready-to-use homologous products. In addition, the use of these materials is limited due to performance and method of use problems associated with the variability of properties of the autologous plasma.

These problems have led to the development of hemostatic compositions which are based on the use of polymeric materials as adhesives. For example, synthetic polymerizable compositions such as cyanoacrylates have been used in adhesive compositions (Ellis, et al., *J. Otolaryngol.* 19:68-72 (1990)). However, the toxicity of many of these synthetic polymer compositions has limited their usefulness.

Natural substances can also exhibit bonding characteristics. In particular, collagen has been reported as being useful as a hemostatic agent (U.S. Pat. No. 4,215,203.) However, when compared with either collagen or synthetic polymers, adhesives which

depend on fibrin exhibit enhanced hemostasis (Raccula, et al., *Am. J. Surg.* 163(2): 234-238 (1992)).

Thrombin-based formulations for use as hemostatics have also previously been described. See, for example, U.S. Patent No. 2,433,299 and 4,363,319. However, the use of thrombin in hemostatic formulations is limited by its instability during storage. Accordingly, many investigators have turned to the use of thrombin in dry form, or its use in conjunction with a substrate such as a solid matrix. In particular, U.S. Patent No. 4,515,637 describes the use of thrombin and collagen in the formation of sponges for use in treating wounds. Additionally, U.S. Patent No. 5,464,471 describes the formation of dry thrombin formulations which are used in conjunction with fibrin monomers.

Recent developments have also led to the production of thrombin - fibrin compositions, which are formulated and used either as "dual component" compositions, or "single component" compositions. In either case, such compositions are sometimes referred to as "fibrin glue", with the thrombin functioning as the "catalyst" component of the glue, and the fibrin functioning as the "resin" component of the glue. Dual-component compositions generally involve the use of separate fibrinogen-containing and thrombin-containing components which are mixed together shortly before or simultaneously upon administration. See, for example, U.S. Patent No. 5,290,552, which describes a dual-component composition that may optionally contain collagen in the fibrinogen-containing component. Single-component compositions generally include both a source of thrombin and a source of fibrin.

In either the single-or dual-component compositions, the fibrin is usually supplied in the form of fibrinogen, which is then converted to fibrin by thrombin. Although the single-component systems are more convenient to use, these compositions generally require the thrombin to be maintained in an inactive state prior to use to prevent premature coagulation. This problem has been addressed in the following two U.S. Patents: U.S. Patent No. 5,318,524 describes the development of single-component hemostatic compositions containing thrombin which has either been inactivated or physically separated (using different phases) from fibrinogen prior to use; and U.S. Patent No. 5,407,671 describes a single-component composition containing a thrombin inhibitor along with the thrombin.

The present invention relates to thrombin-based hemostatic compositions which can reproducibly and effectively be used as a single-component, or in conjunction with a source of fibrinogen, such as autologous plasma. By employing microfibrillar collagen as an additional component in the compositions, many of the aforementioned shortcomings of other hemostatic agents can be avoided.

DISCLOSURE OF THE INVENTION

In accordance with the present invention, it has been discovered that particularly efficacious hemostatic compositions can be formulated by combining thrombin together with microfibrillar collagen. The microfibrillar collagen acts to improve the functionality of the compositions, and also plays an important role in the kinetics of fibrin clot formation, as well as the overall physical characteristics of the fibrin clot.

The thrombin can be derived from a variety of natural sources, such as from pooled animal plasma. For example, bovine thrombin is readily available from a wide variety of commercial sources. In addition, recombinant thrombin can be utilized, which can be synthesized from a variety of recombinant sources such as transformed host (bacterial, yeast or mammalian) cells. In addition, thrombin-like compounds such as certain proteolytic snake venoms, as well as thrombin precursors such as prothrombin can also be used as the source of thrombin for use in the present invention.

The microfibrillar collagen which is useful in the present invention preferably has an average fibril diameter of about 3 to 30 nm. As such, its melting temperature as determined using differential scanning calorimetry is between about 42 to 46°C.

In one embodiment of the present invention, the hemostatic composition further comprises calcium ions, which are added to increase the effective calcium ion concentration at the site of application to an amount which will provide for adequate clot formation. When used at sites having sufficient endogenous calcium ions present, it is not necessary for calcium ions to be added. However, calcium ions are effective at promoting microfibril formation from fibrillar collagen. Accordingly, if the compositions of the present invention are formed from fibrillar collagen, it is desired to add sufficient calcium ions to the composition to form microfibrillar collagen.

The hemostatic compositions of the present invention contain thrombin which remains sufficiently active to be useful for the intended application. Stability depends on

various factors, such as temperature and the presence/absence of added means for stabilizing or destabilizing thrombin. However, it is preferable that the thrombin retains 80% or more of its activity at 2-8°C for at least 6 months.

In a preferred embodiment of the compositions of the present invention, PEG is included to stabilize thrombin. The PEG can be of any suitable molecular weight, such as about 1,000 to 8,000. Additionally, the PEG can be present in a wide range of concentrations, such as 0.1 to 2%, but is preferably present at a concentration of 0.1 to 0.3%.

Since it is desirable to maintain the stability of thrombin upon storage of the hemostatic compositions of the present invention, a further aspect of the present invention involves employing means for stabilizing the thrombin, which can include decreasing the pH to below 6.6, adding a sugar, adding PEG, adding a reversible thrombin inhibitor, adding a calcium chelator and adding a protein.

Another aspect of the present invention is a kit which comprises a thrombin plus microfibrillar collagen containing composition and a source of fibrinogen. The fibrinogen can be added directly to the thrombin-containing composition, or it can be supplied as a separate component in an aqueous medium. A preferred aqueous medium comprising fibrinogen is plasma, and more preferably, it is human plasma, and most preferably it is autologous plasma. In another aspect of the kits of the present invention, a suspension of platelets can be used in conjunction with the thrombin-containing composition, since platelets are activated by thrombin, which initiates the clotting cascade and can effect fibrin clot formation in the absence of exogenously supplied fibrinogen.

In yet another aspect of the present invention, a method for promoting hemostasis of a tissue site on or in the body of a mammalian subject is provided, which entails providing a thrombin-containing composition which additionally contains microfibrillar collagen as one component, and providing a separate fibrinogen solution in an aqueous medium as another component, mixing these two components immediately before contact with the tissue site, then contacting the mixture with the tissue site. In a preferred embodiment of this method, the fibrinogen is supplied in the form of plasma, and more preferably autologous plasma which involves the further step of separating the plasma from the subject's blood.

The method additionally involves the use of a delivery device which is designed for administering multi-component compositions to a treatment site. Such delivery devices can provide for mixing of the two components immediately prior to their being delivered out of the device, or they can alternatively provide for mixing of the two components after exiting the device, such as when sprayed from the device, but before contacting the tissue site.

In still another aspect of the present invention, a method for making hemostatic agents is provided, which comprises the steps of combining together, in no particular order, thrombin, fibrillar collagen and a fiber disassembly agent which is present in sufficient quantities to cause the fibrils to disassemble into microfibrils, and incubating the mixture for an adequate length of time and temperature for the microfibrils to form and become stable. In a preferred embodiment of this method, the fibril disassembly agent is calcium ions, which are preferably present at a concentration of between 10 and 200 mM.

Another embodiment of the present invention relates to compositions comprising collagen that is in the form of a mixture of microfibrillar and non-microfibrillar fibrillar collagen. What is intended by the term non-microfibrillar fibrillar collagen is collagen having fibrils that are larger than microfibrils. In particular, compositions comprising mixtures of microfibrillar collagen and non-microfibrillar fibrillar collagen in a ratio of at least 1:1 (w/w), respectively, may be useful for certain applications. For example, mixtures of microfibrillar collagen and fibrillar collagen with larger fibril sizes may be more flowable and thus easier to use in spraying apparatuses.

Other aspects of the present invention are provided throughout the specification, and although not specifically mentioned in this section, they are considered part of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow chart showing the blood coagulation cascade. (From Enzyme Research Laboratories, Inc., South Bend, IN.) The abbreviations for the various blood coagulation factors are included in TABLE I.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention relates to the use of thrombin-based compositions to promote hemostasis. More particularly, the thrombin-based compositions comprise thrombin and microfibrillar collagen.

- 5 Blood coagulation is a complex cascade of events that results in formation of fibrin strands. Figure 1 illustrates the blood coagulation cascade. The various factors, their molecular weights and typical plasma concentrations are given in TABLE I.

TABLE I

Plasma Concentrations of Blood Coagulation Factors

Component	Molecular Weight (daltons)	Plasma Concentration ($\mu\text{g/ml}$)	Plasma Concentration (μM)
Fibrinogen (I)	330,000	3000	9.09
Prothrombin (II)	72,000	100	1.388
Factor V	330,000	10	0.03
Factor VII	50,000	0.5	0.01
Factor VIII	330,000	0.1	0.0003
Factor IX	56,000	5	0.08928
Factor X	58,800	8	0.13605
Factor XI	160,000	5	0.031
Factor XII	80,000	30	0.375
Factor XIII	320,000	10	0.03124
Protein C	62,000	4	0.0645
Protein S	69,000	10(free)	0.1449
Protein Z	62,000	2.2	0.0355
Prekallikrein	86,000	50	0.5814
HK	110,000	70	0.6363
Fibronectin	450,000	300	0.6667
Antithrombin III	58,000	290	5
Plasminogen	90,000	216	2.4
Urokinase	53,000	0.1	0.001887
Heparin Cofactor II	66,000	90	1.3636
Alpha ₂ -Antiplasmin	63,000	60	0.9524
Protein C Inhibitor	57,000	4	0.0702
Alpha ₂ -Macroglobulin	725,000	2100	2.8966

Thrombin

The primary role of thrombin in the coagulation pathway is its ability to catalyze the conversion of fibrinogen (which may already present at the site of application and/or may be exogenously supplied) to fibrin monomers. Thrombin also plays other important
5 roles in coagulation. For example, thrombin also functions to induce coagulation by activating platelets, which triggers the coagulation cascade. In addition, thrombin catalyzes the conversion of factor XIII to its active form, factor XIIIa, which in turn brings about the covalent crosslinking of the fibrin clot.

Once formed, fibrin monomers aggregate together to form a fibrin clot. The clot
10 can then be further stabilized by covalent crosslinking via factor XIIIa. The microfibrillar collagen which is included in the compositions and thus present during this process of clot formation becomes an integral part of the fibrin clot and thereby alters its properties in a beneficial fashion.

The amount of thrombin which is necessary in the compositions of the present
15 invention is an amount which is sufficient to catalyze the formation of a stable fibrin clot. A clot is considered stable once the formulation is converted from a soft, paste-like liquid to a rubbery gel. This change can be quantified using rheological measurements as described below under Examples. Preferably, the thrombin is present in the hemostatic composition at a concentration of about 10 to 2,000 National Institute of Health (NIH)
20 units/ml, and more preferably from about 300 to 1,000 NIH units/ml.

Thrombin activity, i.e. its efficiency in forming fibrin from fibrinogen, can be determined using a commercially available fibrometer, and comparing the rate of fibrin clot formation using test preparations to those having known thrombin activity. However, thrombin is still suitable for use in the hemostatic compositions of the present invention,
25 even if it does not function well in this test, provided it is still capable of activating platelets. This is because platelet activation catalyzes the formation of a stable clot at an actively bleeding application site via the normal functioning of the coagulation cascade, which is not dependent on exogenously supplied thrombin for fibrinogen cleavage.

Thrombin can be derived from a variety of natural sources. In particular, bovine
30 thrombin is commercially available in lyophilized form (Parke-Davis, Morris Plains, N.J.; GenTrac, Inc., a subsidiary of Jones Medical Industries, Inc., Middleton, WI). Thrombin can also be purified from a source of prothrombin complex as described by Chabbat, et al.,

Thrombosis Research, 76(6): 525-533 (1994). In addition, thrombin can also be derived from recombinant sources. For example, Jorgensen, et al. have described an expression system for gamma-carboxylated recombinant prothrombin (*J. Biol. Chem.* 262:6729-6734 (1987)).

5 Truncated and mutated forms of prothrombin can also be prepared and utilized in the present invention. Le Bonniec, et al. describe cloning of pre-thrombin-2 cDNA (pre-thrombin-2 is a pro-thrombin minus residues 1-155 out of 579 total residues) and cloning and expression of a single amino acid replacement mutant (*J. Biol. Chem.* 266:13796-13803 (1991)). Other references which describe mutated or truncated prothrombins are:
10 Ye, et al., *J. Biol. Chem.* 269:17965-17970 (1994); Rezaie, et al., *Biochemistry* 35:1918-1924 (1996); U.S. Pat. No. 5,476,777 (1995); and Wu, et al., *PNAS* 88:6775-6779 (1991). When the recombinant product is prothrombin, conversion to thrombin can be achieved using a complex of coagulation factor Va, factor Xa, and synthetic phospholipids, or with certain snake venom proteases.

15 Although not preferred, it is possible to use thrombin precursors (e.g. prothrombin) in the hemostatic compositions of the present invention, along with means for conversion to thrombin if such means are not already present at the application site. In addition, thrombin-like compounds such as snake venom proteases which are also capable of forming fibrin clots from fibrinogen are also contemplated by the present invention. (See,
20 e.g. Damus, et al., *J. Lab. Clin. Med.* 79:906-923 (1972)). Accordingly, the term "thrombin" as used herein includes thrombin precursors and thrombin-like compounds, and refers to any protein or amino acid polymer derived from natural or synthetic sources which is capable of catalyzing the formation of fibrin clots from fibrinogen and/or activating platelets.

Collagen

25 The presence of collagen in the hemostatic compositions of the present invention serves to enhance both the mechanical and physiological properties of these compositions. For example, the collagen serves to increase the viscosity of the compositions, which
30 makes them easier to apply to a site without exhibiting run-off. This acts to control the thrombin concentration at the site of application. Additionally, collagen plays a structural

role in clot formation by serving to reinforce the clot. Collagen also serves as an activator of blood platelets which can further promote hemostasis.

Collagen has long been recognized as a component of biomaterials for medical and surgical use. See, e.g., Keefe, et al., *Clinical Materials* 9:155–162 (1992). Collagen can exist in non-fibrillar or fibrillar form. Non-fibrillar collagen consists of triple helices which are approximately 300 nm long and 1.5 nm in diameter (Piez, *Encyclopedia of Polymer Science and Engineering*, Vol. 3, 2nd Ed., John Wiley 699–727 (1985)). In comparison, fibrillar collagen may exist in a wide array of fibrillar sizes. The smallest fibrils arise when 5 to about 30 triple-helical molecules associate. Such associated fibrils have diameters between about 3 to about 30 nm and are roughly 400–500 nm in length. These are generally referred to as “micro-fibrils,” (Gelman, et al., *J. Biol. Chem.* 254:11741–11745 (1979).) Larger fibrils, ranging from 30 to 1000 nm in diameter, and being about 1000 to 5000 nm in length, can also be prepared (Capaldi, et al., *Biopolymers* 21:2291–2313 (1982)).

Collagen fibril size can easily be determined using differential scanning calorimetry (DSC) to measure melting temperatures. In general, non-fibrillar collagen melts at 39–40°C, whereas microfibrillar collagen melts at 42–46°C. See, for example, Wallace, et al., *Biopolymers* 25:1875–1893 (1986).

Collagen fibrils are not readily soluble in aqueous medium and instead form particulate suspensions when added to an aqueous medium. However, as used herein, the term “collagen solution” is not intended to exclude such suspensions of fibrillar collagen. The term “fibrillar collagen” as used herein is meant to refer to collagen in fibrillar form, either micro-fibrils or large fibrils. Additionally, a collagen suspension that consists mostly of micro-fibrils (80% or greater) is referred to herein as a “microfibrillar” collagen suspension.

The viscosity of collagen solutions is influenced by collagen fiber size. For example, solutions of non-fibrillar collagen are more viscous than suspensions of fibrillar collagen (Wallace, et al., *J. Biomed. Mater. Res.* 21: 861–880 (1987).) Additionally, suspensions of smaller fibrils of collagen (i.e., microfibrillar collagen) are more viscous than suspensions of larger fibrils of collagen. As described therein, there are two reasons why microfibrillar collagen suspensions are more viscous than suspensions of collagen with larger fibrils. First, the viscosity of fibrillar collagen suspensions is proportional to

the number density of the fibrils. Since microfibrils are smaller, at the same percentage of total solids, the number density of a microfibrillar collagen suspensions is greater than that of a suspension of collagen having larger fibrils.

Second, the viscosity of rod-like suspensions is dependent on the length-to-width ratio, such that rods with greater length-to-width ratios tend to be more viscous. Doi, et al., *J. Chem. Soc. Faraday II*, 74:560-570 and 918-932 (1978). Micro-fibrils are known to be more elongated than larger fibrils. For example, it has been reported that micro-fibrils have a length-to-width ratio of roughly 20 to 130, whereas intermediate-sized fibrils have a length-to-width ratio of roughly 6 to 75. See, e.g., Table II of Wallace, et al., *Biopolymers* 25:1875-1893, (1986). Hence, microfibrillar collagen suspensions are more viscous than collagen suspensions consisting of larger fibrils.

Even though solutions of non-fibrillar collagen are also viscous, fibrillar collagen is still preferred because of its role in enhancing platelet activity. As described by Balleisen, et al., collagen must at least be in multimeric form (either as fibrils or a precipitate) to promote platelet aggregation (*Hemostasis* 5:155-164 (1976)). Also described therein is that non-fibrillar methylated collagen precipitates in the presence of plasma proteins and thereafter serves as an effective enhancer of platelet aggregation. Hence, although microfibrillar collagen is preferred in the practice of the present, methylated collagen is also a suitable form of collagen if administered to a site at which its interaction with intrinsic plasma proteins causes it to precipitate. Platelet aggregation can be measured using a commercially available aggregation meter. The aggregation of platelets causes an increase in light transmission, which can be recorded and expressed as the time required to reach one-half the maximum transmission.

Microfibrillar collagen is easily prepared using known methods, and can be used directly to form the hemostatic compositions of the present invention. Alternatively, fibrillar collagen can be used, such that upon further processing and storage of the compositions, the fibrils will become disrupted. In general, there are many types of compounds which are known in the art which disrupt fibril formation, which may also act to convert fibrillar forms to microfibrillar forms. For example, the presence of salts can function to disrupt collagen fibrils. Specifically, Zyderm® (greater than 70% by volume fibrils of greater than 30 nm in diameter, Collagen Corporation, Palo Alto, California), which is 3.5% by weight fibrillar collagen in 130 mM sodium chloride can be added to the

aqueous medium of the hemostatic compositions. When Zyderm® is present at a concentration of 20 mg/ml along with 75 mM sodium chloride, the addition of more salt (20 to 40 mM calcium chloride) can function to disrupt the collagen fibrils upon storage at 5–8°C for 3–5 hours. This results in conversion of the collagen to a microfibrillar form.

Alternatively, 200-500 mM sodium chloride alone can be used to form microfibrillar collagen. See Examples, and in particular TABLE III.

Other disassembly agents which can be included for the purpose of converting fibrillar to microfibrillar collagen include, but are not limited to, glycerol, sucrose and related polyols, such as maltose, sorbitol and mannitol.

It is also possible to use non-fibrillar collagen as the starting material by including a compound such as PEG at an approximate concentration of 3-8%, which acts to form microfibrillar collagen from nonfibrillar collagen. Other compounds which can form microfibrillar collagen from non-fibrillar collagen are also well known in the art.

As discussed throughout the specification, and in particular in the Examples below, the concentration and size of collagen fibrils in an aqueous medium is influenced by the storage conditions, such as the pH, temperature, the presence and concentration of other components in the medium, etc. Such conditions can easily be controlled and adapted to optimize the desired collagen fibril characteristics using the teachings of the present specification in combination with well known principles of collagen chemistry. For example, a mixture of microfibrillar collagen and non-microfibrillar fibrillar collagen may result from storage of microfibrillar collagen at room temperature in the presence of compounds known to cause collagen fibril formation. However, as long as the conditions are properly controlled, such aggregation may not necessarily proceed to the point at which the collagen is less than half microfibrillar (which would not be desirable), and may even be desired to make the composition more flowable, since larger fibrils tend to be less viscous.

Calcium Ions

A source of calcium ions is necessary to promote clot formation. As described above, calcium ions can also promote disassembly of fibrils, which can be used to form microfibrils from a fibrillar collagen. Calcium ions are usually added to the hemostatic composition in the form of calcium chloride. Alternatively, a sufficient level of calcium

may already be present at the application site, in which case addition of calcium ions is not necessary. The amount of calcium ions necessary is an amount which is effective to promote clot formation upon administration. Additionally, if the hemostatic composition is formulated from fibrillar collagen, thus necessitating disassembly of collagen fibrils before application, the amount of calcium ions present will also be that which is necessary to convert the fibrillar collagen to the microfibrillar form.

It is important to note that, at certain relative concentrations, the presence of sodium chloride can interfere with fibril disassembly via calcium ions. For example, at 40 mM calcium chloride and 30 mM sodium chloride, microfibrillar formation is inhibited. This may be due to the opposite charge on the calcium and chloride ion, which may lead to ion-ion charge complexes, which in turn lower the activity of either ion relative to collagen. If calcium chloride is present alone, or if calcium chloride and sodium chloride are present at higher concentrations (e.g., 100 mM calcium chloride and 30 to 130 mM sodium chloride or 40 mM calcium chloride and 150 mM sodium chloride), fibril disassembly is not inhibited.

In determining the amount of calcium ions to include in the hemostatic compositions of the present invention, it is also necessary to consider the presence of other constituents in the fibrinogen source or at the site of application. For example, if the hemostatic composition is formulated for use as a two-component composition (as more fully described below) with plasma as the second of the two components, it should first be determined whether the plasma will be derived from a source already containing calcium ions, or from a source which is depleted of calcium. In particular, citrated plasma is a convenient source of plasma, and citrate is an effective calcium chelator which will prevent the availability of calcium for participation in clot formation. If this is the case, then the desired total effective (nonchelated) calcium concentration should be determined, and the appropriate amount of calcium should be added to achieve this total effective concentration.

Thrombin Stabilizers

In a preferred embodiment of the present invention, the thrombin is stable (i.e. it remains active) during storage in an aqueous medium. In particular, the thrombin is preferably stable for at least 30 days at 18-22°C, and more preferably for at least 60 days at

18-22°C. It is well known that stability of pharmaceutical compositions at a lower temperature can be estimated by evaluating their stability at higher temperatures. *See, e.g.,* Segel, et al., *Enzyme Kinetics*, John Wiley & Sons, New York, pages 932-941 (1975.) In the particular case of thrombin-containing compositions, it has been determined that the stability at 18-22°C is roughly one tenth to one twelfth of the expected stability at 2-8°C. *See, e.g.,* European Patent Application 478,827 A1. Accordingly, the hemostatic compositions of the present invention, having been demonstrated to be stable for at least one month at 18-22°C are expected to be stable for at least 10 months at 2-8°C, and are preferably stable for at least 6 months at 2-8°C. It has also been determined that compositions comprising aqueous suspensions of mixtures of microfibrillar and non-microfibrillar fibrillar collagen are stable for at least 12 months at 2-8°C.

Thrombin is known to exhibit autocatalysis brought about by thrombin-thrombin interactions. Accordingly, in order to ensure that the thrombin remains functional, the aqueous medium is preferably maintained at a low pH, in which case thrombin activity is inhibited. For example, the thrombin-containing component of the present invention is maintained at a pH preferably below about 6.6, and more preferably below about 5.7, and is formulated in a manner that will allow the pH to increase upon delivery or at the application site. Other substances which can be added to the aqueous medium to help stabilize thrombin are as follows:

TABLE II
Thrombin Stabilizers

Stabilizers Added	Reference
Bovine serum albumin PEG, 8000 MW	Rotoli, et al., <i>Haematologia</i> 71:463-466 (1986)
Gelatin Polybrene (1,5 dimethyl-1.5 di-aza- undecamethylene poly methobromide)	Topfer, et al., EPO 0478 827 A1 (1990)
PEG, 6000 MW Bovine serum albumin	Oshima, et al., <i>Thrombosis Res.</i> 47:59-67 (1987)

TABLE II
Thrombin Stabilizers

Stabilizers Added	Reference
Glycerol Ethylene glycol PEG, 6000 MW	LeBorgne, et al., <i>Applied Biochem. Biotechnol.</i> <u>48</u> :125-135 (1994)

The inclusion of hydrophilic polymers such as polyalkyl oxides, preferably polyethylene glycol (PEG), in the thrombin-based hemostatic compositions serves to stabilize the thrombin by inhibiting thrombin-thrombin interactions, which prevents autocatalysis. PEG can be added to the hemostatic formulation in admixture, or it can be premixed with thrombin. However, the addition of excess PEG favors formation of fibrillar collagen and should be avoided. The amount of PEG to be added is easily determined by adjusting the concentration to optimize stabilization and fiber size. Generally, PEG is present below 2%, and is preferably below 1%, and more preferably approximately 0.1 to 0.3%.

Additional stabilizers include sugars (e.g. sorbitol, mannitol, glucose, sucrose) and amino acids (e.g. glycine) as described in EPO 478,827A1; EPO 302,754B1; and GB2,041,942A. Still other means for stabilizing thrombin are described in U.S. Pat. Nos. 5,219,328; 5,318,524; and 5,407,671.

Biologically Active Agents

In addition to their role in hemostasis, the compositions of the present invention may serve as effective delivery vehicles for a wide variety of components that have various desirable physical, chemical, biological and/or therapeutic effects at the site of application or on the body of the subject. For example, antibiotics can be supplied to the application site to prevent infection. Such antibiotics are well known, as are their dosage amounts and means of incorporation. Additionally, antifibrinolytic agents such as aprotinin, C1-esterase inhibitor and ϵ -amino-n-caproic acid can be included to enhance clot integrity.

It is also possible to include one or more growth factors in the hemostatic composition to promote wound healing at the site of application, such as platelet-derived growth factors, insulin-binding growth factors, fibroblast growth factors, transforming

growth factors, platelet factors and heparin binding growth factors. See, for example, *Growth Factors and Other Aspects of Wound Healing: Biological and Clinical Implications*, Alan R. Liss, Inc., New York, New York, pp. 303-329 (1988).

The incorporation of biologically active agents such as drugs into tissue sealant compositions has already been extensively described. See, for example, PCT WO 96/17633. Virtually any biologically active agent which can be incorporated into such a tissue sealant can also be incorporated into the hemostatic compositions of the present invention.

Formulation

The hemostatic compositions of the present invention are formulated for administration in an aqueous medium which may optionally contain buffers, viscosifiers, osmolality enhancers and other substances that are desired and/or necessary to assure biocompatibility, injectibility and efficacy. Depending on the particular application, the compositions can be formulated in various different configurations. For example, a single-component composition containing thrombin and microfibrillar collagen in an appropriate aqueous medium can be utilized for effecting hemostasis at an application site that already has a source of fibrinogen, along with the other clotting factors and conditions which are necessary for the conversion of fibrinogen to fibrin. In other instances, for example if the site of application is not an actively bleeding site, it may be necessary to supply a source of fibrinogen (and any other clotting factors which may be deficient at the treatment site), either in the same single-component composition or as a separate component. However, when thrombin and fibrinogen are included in the same single-component composition, it is necessary to maintain thrombin in an inactive state such that it will be activated upon application.

In two-component compositions, one of the components will necessarily contain thrombin and microfibrillar collagen, and the second component will contain a source of fibrinogen. Although not preferred for lack of convenience, it may also be desired to supply calcium in a separate third component, since calcium ions are necessary for various hemostatic functions, including activation and function of factor XIII, platelet functions, such as aggregation, and finally, for further production of active clotting factors in blood. Accordingly, the absence of calcium may help maintain thrombin stability. In either case,

it is preferable for the collagen to be supplied separately from the fibrinogen component, so that during application adequate mixing can be achieved between the thrombin and the fibrinogen.

The fibrinogen source can be synthetic or natural. For convenience, plasma can be used as the fibrinogen source. Preferably, the plasma is human, and more preferably autologous plasma, which is an effective way of avoiding the transfer of infectious agents and other undesirable constituents sometimes found in pooled plasma. The autologous plasma is generally harvested from citrated (.32-.38% w/v) blood, which is preferably drawn within a few hours before use. Platelet-rich plasma is separated from the blood using known methods, for example, by centrifugation at 90 x G for 10 min., or at 1000 x G for 3 min.

Given below are various alternative multi-component embodiments which may be combined together for use in various volume:volume ratios of A:B, such as 0.4:1.0, 1.0:1.0, 1.0:0.4, and 1.0:2.0, with 1:1 being preferred.

Two-Component Composition (1):

Component A: Type I fibrillar collagen (20 mg/ml)
Calcium chloride (40 mM)
Thrombin (at least 500 NIH units/ml)
Sodium chloride (150 mM)
PEG (0.2% w/v, 1000 MW)
Mannitol (15 mM)
pH 4.7-6.6

Component B: Platelet-rich plasma in 0.32-0.38% sodium citrate

Two-Component Composition (2):

Component A: Type I fibrillar collagen (20 mg/ml)
Calcium chloride (20 mM)
Thrombin (at least 500 NIH units/ml)
Sodium chloride (300 mM)
PEG (0.2% w/v, 1000 MW)
Mannitol (15 mM)
pH 4.7-6.6

Component B: Human fibrinogen 5 mg/ml
in 20 mM sodium chloride
10 mM sodium phosphate, pH 7.5
15 mM mannitol

5

Two-Component Composition (3):

Component A: Type I fibrillar collagen (20 mg/ml)
Calcium chloride (20 mM)
Thrombin (at least 500 NIH units/ml)
Sodium chloride (300 mM)
PEG (0.2% w/v, 1000 MW)
Mannitol (15 mM)
pH 4.7-6.6

10

Component B: Plasma depleted of fibrinogen by heat, or serum, plus blood
platelets at $2-4 \times 10^8$ /ml

15

Two-Component Composition (4):

Component A: Type I fibrillar collagen (20 mg/ml)
Calcium chloride (20 mM)
Thrombin (at least 500 NIH units/ml)
Sodium chloride (300 mM)
PEG (0.2% w/v, 1000 MW)
Mannitol (15 mM)
pH 4.7-6.6

20

Component B: Blood platelets at $2-4 \times 10^8$ /ml in isotonic saline plus suitable
stabilizer excipients for platelets.

25

In an alternative embodiment, the following three-component composition can be used, with each of the three components, A:B:C, being mixed in a volume ratio of approximately 0.4-2.0:0.4-2.0:0.4-2.0, with 1:1:1 being preferred.

Three-Component Composition:

5 Component A: Type I fibrillar collagen (30 mg/ml)
 Calcium chloride (40 mM)
 PEG (0.2% w/v, 1000 MW)
 Component B: Thrombin (at least 1000 NIH units/ml)
 Sodium chloride (370 mM)
10 Mannitol (15 mM)
 pH 4.7-6.6
 Component C: Platelet-rich plasma in 0.32-0.38% sodium citrate

In a more preferred three component composition, the PEG is included with
15 Component B containing the thrombin to facilitate stabilization of the thrombin as follows:

Three-Component Composition (2):

 Component A: Type I fibrillar collagen (30 mg/ml)
 Calcium chloride (40 mM)
 Component B: Thrombin (at least 1000 NIH units/ml)
20 Sodium chloride (370 mM)
 PEG (0.2% w/v, 1000 MW)
 Mannitol (15 mM)
 pH 4.7-6.6
 Component C: Platelet-rich plasma in 0.32-0.38% sodium citrate

25 Testing of Clot Formation

The hemostatic compositions of the present invention interact with fibrinogen to form a gelatinous clot at the site of application. This clot acts as a physical barrier to blood flow. Thus, the efficiency of these compositions is proportional to the strength of the clots they can form. In addition, the thrombin in the composition clots any blood which is
30 present at the site, which adds to the efficiency of the composition to promote hemostasis.

The clotting efficiency of the hemostatic compositions can easily be determined by observing instantaneous clot formation upon administration. However, in order to compare the efficiency of clot formation of various different formulations for purposes of optimization, such comparisons can be made on the basis of rheometric measurements which are taken during clot formation. As described by Rosenblatt, et al. (*J. Appl. Polym. Sci.* 50: 953-963 (1993)), the dynamic elastic modulus, G' , and the dynamic viscous modulus, G'' , are determined as a function of the elasticity and the overall strength of a gel, respectively. (See, also, Ferry, *Viscoelastic Properties of Polymers*, 3d ed., John Wiley, New York, pages 1-31 and 41-44 (1980); and Janmey, et al., *Blood*, 80(4): 928-936 (1992)).

Use and Administration

The compositions of the present invention are useful in many different applications where hemostatic agents, tissue sealants and tissue adhesives are normally used. The present invention is especially suitable for stopping diffuse capillary bleeding, for example in parenchymal organ bleeding, such as that of the liver, spleen and kidney. In addition, these compositions are useful in treating bleeding associated with debridement during healing of burned tissues.

In one aspect of the present invention, the compositions are useful in controlling diffuse bleeding from cancellous bone surfaces, which are highly vascular and thus prone to excessive post-surgical bleeding. Such bleeding can pose problems during a variety of different surgical procedures, such as in the field of orthopedics, neurosurgery, plastic and reconstructive surgery, spinal surgery and oral-maxillo-facial surgery. Post-surgical application of the compositions of the present invention can therefore be used to lessen post-surgical blood loss.

The compositions of the present invention are also useful in controlling intraoperative bleeding that has been exacerbated by genetic or acquired clotting defects or the use of anticoagulation therapy. For example, if a patient receives anticoagulant therapy following surgery and subsequently needs additional surgery, the compositions of the present invention are useful in counteracting the increased bleeding caused by the anticoagulants.

The single-component compositions can be administered directly to the treatment site. Multi-component compositions are preferably premixed, i.e. they are mixed either

immediately prior to or during administration, as opposed to being administered in such a manner that all the components come in contact with one another for the first time at the application site. Pre-mixing during administration generally means that the components are either mixed together during delivery while still contained within the delivery device but immediately before exiting the device, or they can be mixed together after exiting the device, but before they contact the application site. In the latter type of device, the contents of two or more compartments can be sprayed from the separate compartments into a single stream of fluid during application. Devices which are suitable for administration of multi-component compositions can be modified to adjust premixing time and efficiency, and are described in U.S. Patent Nos. 5,116,315; 4,874, 368; and 4,359,049.

It is desirable for each of the components of the composition, and especially the collagen-containing component, to be flowable, i.e. to be capable of being administered through a small-gauge orifice, such as a 200-1000 micron opening. To facilitate application by spraying, it is preferable that the compositions be flowable through a 300 micron opening at a flow rate of approximately 1 ml per second. Subsequent gelation allows the compositions to remain in place after application.

The dosage amount will necessarily depend on the particular use for which the composition is being administered, as well as the desired degree of hemostasis, and can be easily determined by a skilled physician.

EXAMPLES

Example 1

Evaluation of Collagen Fiber Size

This experiment was performed to determine the effects of different salt concentrations on the disassembly of collagen fibers. Collagen was purified from bovine corium using pepsin solubilization as described by McPherson, et al., *Collagen Rel. Res.* 5: 119-135 (1985). Following collagen solubilization, fibrillar collagen was reformed by resuspending the soluble (non-fibrillar) collagen in a buffer containing 20 mM sodium phosphate, pH 7.2. The fibers thus formed were harvested by centrifugation and resuspended in water at 20 mg protein/ml.

Several different samples were prepared as indicated below in TABLE III, and the melting temperature of each sample was determined initially after preparation, and after storage for three days at room temperature, using differential scanning calorimetry (DSC). Each sample contained 1-3 mg protein in physiological saline. A total volume of 15 to 30 μ l was sealed in pans and heated at 10°C/min in a Mettler TA3000 calorimeter with DSC20 cell (Hightstown, NJ). A reference sample pan containing the same weight of physiological saline was heated with each sample.

TABLE III
Differential Scanning Calorimetry (DSC)

Sample Composition				Melting Temperature, °C	
Sample No.	Calcium Chloride, mM	Sodium Chloride, mM	PH	0 days	3 days
1	0	2	7.2	56.8	56.3
2	40	2	7.2	48.3	45.0
3	40	30	7.2	50.5	49.0
4	100	30	6.0	43.5	43.5
5	40	150	6.3	48.4	43.8

Fibril size was estimated based on the melting temperature results according to Wallace, et al., *Biopolymers*, 25: 1875-1893 (1986.) In general, melting temperatures for microfibrillar collagen are lower than for fibrillar collagen. A melting temperature of 49-50°C indicates the presence of intermediate-sized fibers, whereas a melting temperature of 44°C indicates the presence of microfibrillar collagen. According to the DSC results, Sample 2, which contained 40 mM calcium chloride and only 2 mM sodium chloride demonstrated intermediate-sized fibrils at time zero, but had converted to microfibrillar form by day three. In comparison, Sample 3, which contained both calcium chloride and sodium chloride did not exhibit a change in fiber size, which may indicate a counteraction of the calcium ion mediated fiber disassembly in the presence of sodium chloride. Sample

4, at a calcium chloride concentration of 100 mM, appears to have undergone fiber disassembly prior to the melting temperature being determined. Sample 5, like Sample 2, exhibited a conversion from intermediate-sized fibrils to microfibrils. In Samples 4 and 5, high levels of both salts appeared to promote fibril disassembly, in contrast to Sample 3.

5

Example 2

Electron Microscopy Studies

Electron microscopy was performed to confirm the state of collagen fibrils in the presence and absence of calcium chloride. In particular, a suspension of fibrillar collagen (20 mg protein/ml) was prepared as described in Example 1. To this suspension, either 25 mM of calcium chloride or an equal volume of water was added. These two test suspensions were incubated at room temperature for 3 weeks to allow disruption of the collagen fibrils.

15

As observed using electron microscopy, the sample without calcium chloride exhibited loosely woven fibrils, both non-banded and banded, which were 100-250 nm in diameter. In comparison, the sample with calcium chloride exhibited microfibrils which were rarely banded, and which were between 3 and about 30 nm in diameter.

20

Example 3

Evaluation of Fibrin Clot Strength

This experiment was performed in order to determine the ability of different hemostatic compositions to form fibrin clots. Samples were prepared as described in Example 1 and used after incubation at three days, with the exception of Sample 3 which was used immediately after preparation. All of the samples, except Sample 1, also contained 500 NIH units/ml thrombin. Clot formation was initiated by mixing the hemostatic compositions with an equal volume of rabbit plasma containing sodium citrate at 0.32%, w/v. (Sample 1 was mixed with an equal volume of water to determine the contribution of collagen alone to the gel structure of the clots.) In order to inhibit premature gelation, the pH of the plasma was acidified to 5.0 before mixing with the sample compositions.

25

30

After mixing, the sample-plus-plasma mixture was loaded into the sample cell, and the pH was adjusted to 7.2. The sample cell was maintained at 25°C, and measurements were taken after two hours. Rheologic measurements were made in a Rheometrics fluid spectrophotometer, Model 8400 (Piscataway, NJ). Measurements were taken in dynamic mode in a parallel plane geometry. Gelation was monitored at a 1 radian/sec. oscillation rate and 1% strain, which would not be expected to deform the gel structure. As an indication of the susceptibility of the gel to total breakdown, additional testing was performed at 50% strain.

TABLE IV

Rheometry

Sample Composition			Results		
Sample No.	Calcium Chloride, mM	Sodium Chloride, mM	G' at 1%, dynes/cm ²	G'' at 1%, dynes/cm ²	G' at 50%, dynes/cm ²
1	0	74	110	46	34
2	40	52	210	17	480
3	40	150	310	26	830
4	40	150	570	66	1040
5	114	126	1010	130	1300

Sample 1, which contained no thrombin or fibrinogen, exhibited a G' value which was indicative of a material with only modest viscous properties. Additionally, as indicated by the decrease of G' at 50% strain, the gel was completely broken down. In contrast, all other samples exhibited a higher G' at 1% strain, with no decrease in G' at 50% strain. These results also indicate a relatively low G'', which is indicative of an elastic solid with little viscous character.

Samples 3 and 4 differed only in that Sample 3 was prepared immediately before mixing with plasma, and Sample 4 was stored for three days prior to use. This difference would be expected to demonstrate the effect of fibril size on gel elasticity and strength, Sample 3 having intermediate-sized fibrils and Sample 4 having microfibrils as described

in Example 1. As expected, the presence of microfibrils in Sample 4 resulted in formation of a clot with improved elasticity and strength.

In Sample 5, high levels of both salts appear to be able to disassemble fibrils to form micro-fibrils. This is in contrast to an apparent "canceling" effect of calcium and sodium chloride at about 30 mM, as noted before.

Also observed is the direct relationship between the salt concentrations and G' which, when considered in light of the results given in Example 1, indicates that the presence of microfibrillar collagen results in a more elastic clot. Such a clot is expected to be better able to resist displacement by blood flow or other forces that would cause it to be dislodged from the site of application.

Example 4

Effect of PEG on Thrombin Stability

The optional inclusion of PEG enhances the stability of the thrombin in the hemostatic compositions of the present invention. In order to evaluate the differences between PEG of varying size, and at varying concentrations, thrombin activity was evaluated at different time periods after incubation at 18-22°C. Each sample contained Zyderm® Collagen (Collagen Corporation, Palo Alto, CA), 40 mM calcium chloride (to convert fibrils to microfibrils) and 500 NIH units/ml thrombin (GenTrac, Inc., Middleton, WI), at a pH of 6.4. Thrombin activity was determined using a BBL Fibrosystem fibrometer and an appropriate fibrinogen control, such as Coagulation Control Level 1 (Sigma Chemical Co., Saint Louis, MO). A 200 ml aliquot of the fibrinogen control was pre-incubated for 2 min. at 37°C in the reaction cup, and the reaction was initiated by adding 100 ul of the sample to be tested, after dilution to approximately 5 NIH units/ml with 20 mM HEPES, pH 7.4, 40 mM CaCl_2 . The fibrometer recorded the time to first fiber formation, which was used to determine thrombin concentration from comparison to a standard curve. Thrombin activity is expressed as a percent of the initial thrombin activity. Thrombin activity after 12 days at room temperature as a function of concentration and molecular weight is presented below in TABLE V.

TABLE V
Effect of PEG on Thrombin Stability

	Average Molecular Weight of PEG*		
Concentration	1000	3350	8000
2%	69	48	20
1%	60	42	29
0.5%	64	47	22
0.2%	90	82	32

* The 1000 and 8000 molecular weight PEG was obtained from Sigma Chemical Company, St. Louis, MO. The 3350 molecular weight PEG was obtained from Union Carbide Chemical Company, South Charleston, WV.

As shown by these results, thrombin activity was greatest at the lowest level of PEG, and using the smaller molecular weight (1000 and 3350) PEG. In a subsequent experiment, aliquots of the sample containing 0.2 % PEG-1000 containing 0.02% sodium azide were adjusted to various pHs, between 4.0 and 6.4. The sample at pH 4.0 lost all thrombin activity. The other samples retained over 70% of their activity for up to 45 days.

Later experiments investigated the effect of a lower concentration of PEG-1000 (0.1%), no PEG, PEG-400 (0.2%), PEG-3350 (0.2%) and PEG-8000 (0.2%), all containing 0.02 % sodium azide. Each of these samples retained at least 50% of thrombin activity for up to 30 days.

Example 5

Effect of Microfibrillar Collagen on Platelet Aggregation and Hemostasis

Platelet aggregation was performed with a Model DP-247 E dual Sample Aggregation Meter (Sienco, Inc., Morrison, Company). The scale was adjusted using platelet poor plasma and platelet rich plasma (PRP) as described in the owners manual. A 360 ul aliquot of PRP was heated at 37°C for 5 min. in a cuvette while stirring. The reaction was then initiated by the addition of 40 ul of the sample. Formulations tested

were as follows: Sample 3, described above in Example 1, TABLE III, after 7 weeks incubation and conversion to a micro-fibrillar state (as determined by DSC melting temperature); and a freshly made sample identical to Sample 3, which contained fibrillar collagen, which was diluted with saline to 1 mg/mL. The aggregation of platelets causes an increase in light transmission which is recorded on a strip chart recorder. Efficiency of platelet aggregation is expressed as the time required to reach one-half the maximum transmission ($T_{1/2}$) Using the sample containing fibrillar collagen, $T_{1/2}$ was 2.1 min., in comparison to 1.4 min. for the sample containing microfibrillar collagen.

These same samples were tested *in vivo* for hemostatic efficiency in an animal model. This study also demonstrated superiority of the sample containing microfibrillar collagen, as indicated by a faster time to achieve hemostasis when compared to the sample containing fibrillar collagen.

Example 6

Platelet Activation

This experiment was performed in order to assess the efficacy of hemostatic compositions of the present invention to form clots in the absence of fibrinogen, but in the presence of platelets. Fresh rabbit blood was centrifuged for 3 minutes at 1380 X g, and the platelet rich plasma was separated. This plasma was centrifuged again for 45 minutes at the same speed to pellet the platelets. The plasma-poor supernatant was heated to between 53 and 56°C for 3 minutes to precipitate the fibrinogen. After removing the fibrinogen by centrifugation, the plasma was returned to the platelet pellet. This fibrinogen-depleted plasma, a sample of normal plasma and a 1:1 mixture of the two were all tested for their ability to form fibrin clots.

Plasma samples (or normal saline as a control) were mixed in a 1:1 ratio with a hemostatic composition containing 20 mg/ml collagen, 500 U/mL bovine thrombin and 40 mM CaCl_2 , and immediately applied to an actively bleeding tissue site in an animal model. The hemostasis times for those sites that stopped bleeding in less than two minutes are shown below in TABLE VI.

TABLE VI
Hemostatic Time

Platelet Level	Fibrinogen Level	Hemostasis Time, seconds	No. Of Sites No Longer Bleeding After 2 min., # / N
Normal	Normal	48	10/10
Normal	50% of Normal	49	10/10
Normal	Depleted	51	10/10
None	None	97	4/10

As demonstrated by these results, even the sample which was combined with plasma depleted of fibrinogen exhibited hemostatic times which were nearly equivalent to those achieved using plasma with normal fibrinogen levels.

Example 7

In Vivo Rabbit Hemostasis

In order to test the ability of the compositions of the present invention to facilitate hemostasis *in vivo*, the following two-component test composition was prepared: component 1) pepsin-solubilized bovine corium type I collagen was prepared as described in Example 1 above to a final concentration of 20 mg/ml collagen, 40 mM CaCl₂ and 500 U/ml bovine thrombin; component 2) rabbit platelet rich plasma, obtained by centrifuging citrated whole blood for 2 minutes at 1380 x g. The two components were mixed during application in a two-compartment spraying apparatus. This sample preparation was compared to a conventional two-component fibrin sealant composition comprising lyophilized human fibrinogen (Hemacure, Kirkland, Quebec, Canada) reconstituted to 100 mg/ml protein in deionized water as the first component, and 500 U/ml thrombin in 40 mM CaCl₂ as the second component. Also studied were results using InstatTM collagen sponges (Johnson & Johnson Medical, Arlington, Texas).

The rabbits were anesthetized using standard procedures, and the kidneys and spleen exposed by performing a midline laparotomy. Incisions were made in the kidneys and spleen (2 mm deep by 15 mm long), and pressurized with gauze prior to application of

the test samples. The time to hemostasis following administration of the test samples was measured, along with the total blood loss which was determined using preweighed cotton swabs or gauze to absorb the blood from the surgical field. Studies were performed (n=5-6) on untreated rabbits, as well as rabbits that had been pretreated with either aspirin or heparin as anticoagulants. The results are reported below in Table VII.

Table VII

In Vivo Hemostatic Performance*

Hemostatic Agent	Normal Coagulation		Aspirin Treated		Heparin Treated	
	Hemostasis Time (sec)	Blood Loss (ml)	Hemostasis Time (sec)	Blood Loss (ml)	Hemostasis Time (sec)	Blood Loss (ml)
Kidney:						
Test Sample	27 ± 2	0.05 ± 0.16	27 ± 4	0.1 ± 0.3	53 ± 15	0.21 ± 0.39
Instat™	78 ± 15	0.79 ± 0.76	96 ± 12	0.8 ± 0.8	70 ± 14	2.0 ± 1.1
Fibrin Sealant	68 ± 13	ND	147 ± 37	1.0 ± 1.6	148 ± 37	2.0 ± 2.0
None	225 ± 16	2.7 ± 1.9	349 ± 42	3.6 ± 3.2	456 ± 51	3.9 ± 2.4
Spleen:						
Test Sample	130 ± 25	1.1 ± 1.6	57 ± 32	0 ± 0	235 ± 53	0.5 ± 0.7
Instat™	122 ± 23	0.9 ± 0.6	139 ± 41	0.5 ± 0.6	378 ± 92	4.6 ± 2.7
Fibrin Sealant	152 ± 38	ND	145 ± 49	0.3 ± 0.4	85 ± 66	0.3 ± 0.6
None	> 600	5.4 ± 3.0	> 600	4.1 ± 3.0	> 600	8.8 ± 3.3

* Measurements are reported ± s.e.m.

As shown above in Table VII, the test sample exhibited shorter hemostasis times and less blood loss than either the fibrin sealant or the collagen sponge in the kidney model. In the spleen model, more variability was observed between animals, so comparisons between treatments may not be accurate. However, in all instances, use of the test sample composition showed a significant improvement when compared to untreated animals.

Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in the field of tissue repair are intended to be within the scope of the following claims. All publications, patents, and patent applications cited in this specification are incorporated herein by reference as if each such publication, patent or
5 patent application were specifically and individually indicated to be incorporated herein by reference.

CLAIMS

What is claimed is:

1. A hemostatic composition comprising thrombin and microfibrillar collagen
5 in an aqueous medium.
2. The composition of claim 1, wherein the thrombin is bovine thrombin.
3. The composition of claim 1, wherein the thrombin is recombinant thrombin.
4. The composition of claim 1, wherein the thrombin is a thrombin-like
compound.
- 10 5. The composition of claim 1, wherein the microfibrillar collagen has an
average diameter of about 3-30 nm.
6. The composition of claim 5, wherein the microfibrillar collagen has a
melting temperature of about 42 to 46°C.
7. The composition of claim 1 further comprising calcium ions.
- 15 8. The composition of claim 7, wherein the calcium ions are present in the
form of calcium chloride.
9. The composition of claim 7, wherein the calcium ions are present at a
concentration which is effective to disassemble fibrillar collagen into microfibrillar
collagen.
- 20 10. The composition of claim 1, wherein the thrombin retains 80% or more of
its activity after storage at 2-8°C for at least 6 months.
11. The composition of claim 1 further comprising PEG.
12. The composition of claim 1, wherein the PEG has an average molecular
weight of about 1,000 to 8,000.
- 25 13. The composition of claim 11, wherein the PEG is present at a concentration
of between 0.1% and 2% w/v.
14. The composition of claim 13, wherein the PEG is present at a concentration
of between 0.1 and 0.3% w/v.
15. The composition of claim 1, wherein the pH of the composition is between
30 4.5 and 6.6.
16. The composition of claim 1, wherein the pH of the composition is between
5.0 and 5.7.

17. The composition of claim 1 further comprising at least one sugar, wherein the sugar is present in an amount which is effective to stabilize the thrombin.

18. The composition of claim 1, wherein the sugar is selected from the group consisting of: mannitol, sorbitol, glucose and sucrose.

5 19. The composition of claim 1 further comprising at least one biologically active agent.

20. The composition of claim 19, wherein the biologically active agent is selected from the group consisting of: growth factors, antibiotics and therapeutic agents.

21. The composition of claim 1, further comprising fibrinogen.

10 22. The composition of claim 1, further comprising at least one means for stabilizing thrombin to a greater extent than that of an equivalent composition not employing said means for stabilizing thrombin.

15 23. The composition of claim 22, wherein said means are selected from the group consisting of: decreasing the pH below 6.6, adding a sugar, adding PEG, adding a thrombin inhibitor, decreasing calcium ion concentration, adding a calcium chelator and adding a protein.

24. The composition of claim 1, wherein the composition is flowable through an opening of about 200 to 1000 microns.

25. A kit comprising the composition of claim 1 and a source of fibrinogen.

20 26. The kit of claim 25, wherein the source of fibrinogen is fibrinogen in an aqueous medium.

27. The kit of claim 25, wherein the source of fibrinogen is plasma.

28. The kit of claim 27, wherein the plasma is human plasma.

25 29. The kit of claim 27, wherein the plasma is autologous plasma.

30. A kit comprising the composition of claim 1 and a suspension of platelets.

31. A method for promoting hemostasis of a tissue site on or in a body of a mammalian subject comprising the steps of:

30 (a) providing a hemostatic composition comprising thrombin and microfibrillar collagen in an aqueous medium;

(b) providing an aqueous medium comprising fibrinogen;

(c) mixing the hemostatic composition and the aqueous medium comprising fibrinogen immediately prior to contact with the tissue site; and

(d) contacting the mixture with the tissue site.

32. The method of claim 31, wherein the aqueous medium comprising fibrinogen is plasma, and the step of providing an aqueous medium comprising fibrinogen further comprises isolating the plasma from the mammalian subject.

33. The method of claim 32, wherein the mammalian subject is a human and the plasma is human plasma.

34. The method of claim 33, wherein the human plasma is isolated from a sample of blood from the human subject.

35. The method of claim 31, wherein the step of mixing takes place in an apparatus during delivery of the hemostatic composition and the aqueous medium comprising fibrinogen to the tissue site.

36. The method of claim 35, wherein the step of mixing takes place after the hemostatic composition and the aqueous medium comprising fibrinogen exit the apparatus, but before the hemostatic composition and the aqueous medium comprising fibrinogen contact the tissue site.

37. A method for promoting hemostasis of a tissue site on or in a body of a mammalian subject comprising the steps of:

(a) providing a hemostatic composition comprising thrombin and microfibrillar collagen in an aqueous medium;

(b) providing an aqueous medium comprising platelets;

(c) mixing the hemostatic composition and the aqueous medium comprising platelets immediately prior to contact with the tissue site; and

(d) contacting the mixture with the tissue site.

38. A method of making a composition for use as a hemostatic agent comprising the steps of:

(a) mixing thrombin, fibrillar collagen and a fiber disassembly agent together in an aqueous medium, wherein the fiber disassembly agent is present at a concentration which is effective at disassembling the fibrillar collagen into microfibrillar collagen; and

(b) incubating the mixture formed in step (a) for a sufficient length of time and at a sufficient temperature to form microfibrillar collagen having a stable fiber size.

39. The method of claim 38, wherein the fiber disassembly agent is calcium ions.

5 40. The method of claim 39, wherein the calcium ions are present at a concentration of between 10 and 200 mM.

41. The method of claim 39, wherein the fiber size of the microfibrillar collagen is between 3 and 30 nm in diameter.

10 42. The method of claim 38, wherein the method further comprises adding fibrinogen to the aqueous medium.

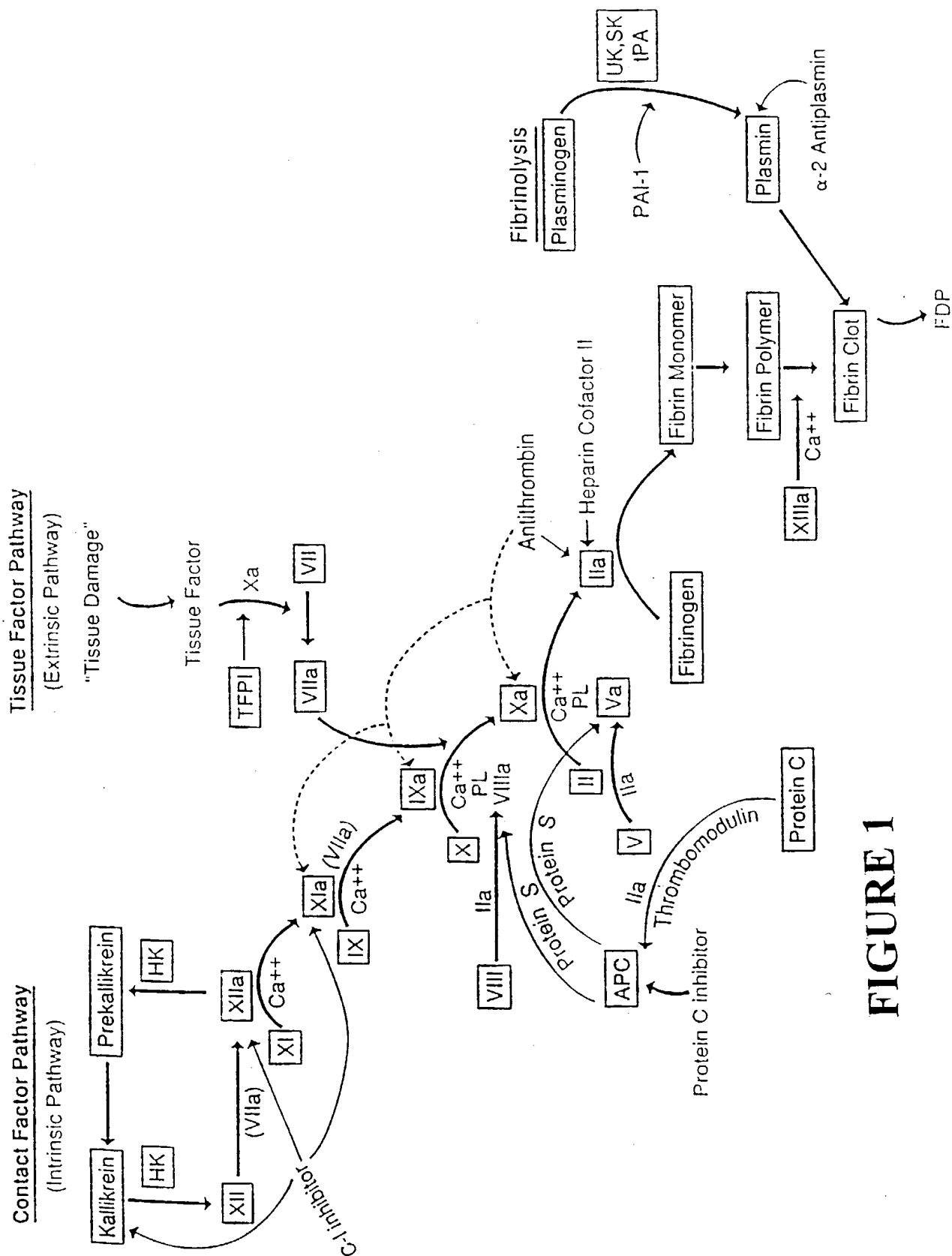
43. A hemostatic composition comprising thrombin and collagen in an aqueous medium, wherein the collagen is a mixture of microfibrillar collagen and non-microfibrillar fibrillar collagen.

15 44. The composition of claim 43, wherein the ratio of microfibrillar to non-microfibrillar fibrillar collagen is at least 1:1 (w/w).

45. The composition of claim 43, wherein the microfibrillar collagen has a fibril diameter of about 3 to 30 nm and a fibril length of about 400 to 500 nm, and wherein the non-microfibrillar fibrillar collagen has a fibril diameter of about 30 to 1000 nm and a fibril length of about 1000 to 5000 nm.

20

1/1





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61L 25/00		A3	(11) International Publication Number: WO 98/57678
			(43) International Publication Date: 23 December 1998 (23.12.98)
(21) International Application Number: PCT/US98/12687 (22) International Filing Date: 17 June 1998 (17.06.98) (30) Priority Data: 08/878,471 18 June 1997 (18.06.97) US 09/099,126 17 June 1998 (17.06.98) US (71) Applicant (for all designated States except US): COHESION TECHNOLOGIES, INC. [US/US]; 2500 Faber Place, Palo Alto, CA 94303 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): PRIOR, Jeffrey, J. [US/US]; 602 Spar Drive, Redwood City, CA 94065 (US). SIERRA, David, H. [US/US]; 48 Middle Gate, Atherton, CA 94027 (US). WALLACE, Donald, G. [US/US]; 290 Ringwood Avenue, Menlo Park, CA 94025 (US). DELUSTRO, Frank, A. [US/US]; 2517 DeKoven Avenue, Belmont, CA 94002 (US). (74) Agents: AXFORD, Laurie, A. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 1 April 1999 (01.04.99)	
(54) Title: COMPOSITIONS CONTAINING THROMBIN AND MICROFIBRILLAR COLLAGEN			
(57) Abstract			
<p>The present invention relates to thrombin-containing hemostatic compositions, their preparation and use. In particular, it relates to hemostatic compositions comprising stabilized thrombin and microfibrillar collagen in an aqueous medium. In a preferred embodiment of the present invention, the compositions are used in a kit comprising two different components, one of which is autologous patient's plasma as the source of fibrinogen, and the other of which is the thrombin-containing composition which also contains microfibrillar collagen.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/US 98/12687

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61L25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 290 552 A (SIERRA DAVID H ET AL) 1 March 1994 cited in the application see column 3, line 44 - line 63 see claims 1,3-5 see examples 1,2 ---	1-10, 15-29, 31-36, 38-45
X	US 4 891 359 A (SAFERSTEIN LOWELL ET AL) 2 January 1990 see column 2, line 57 - column 3, line 34 see column 5, line 64 - column 6, line 19 see claim 8 --- -/--	1-6, 10-20, 22-29, 31-36, 43-45

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

20 January 1999

Date of mailing of the international search report

29/01/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Heck, G

INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/US 98/12687

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>EP 0 341 007 A (PROJECT HEAR; MATRIX PHARMA (US)) 8 November 1989</p> <p>see example 1</p> <p>see claims 2,4</p> <p>-----</p>	<p>1-10, 15-29, 31-36, 38-45</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 12687

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 31-37
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 31-37
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition. (Pls.see R.39.1(iv) PCT)
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 98 12687

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 31-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/12687

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5290552 A	01-03-1994	AT 111360 T	15-09-1994
		CA 1339090 A	29-07-1997
		DE 68918155 D	20-10-1994
		DE 68918155 T	02-03-1995
		EP 0341007 A	08-11-1989
		ES 2064439 T	01-02-1995
		JP 2071747 A	12-03-1990
		JP 2774141 B	09-07-1998
US 4891359 A	02-01-1990	AU 623456 B	14-05-1992
		AU 4603789 A	14-06-1990
		CA 2004739 A	08-06-1990
		DE 68914110 D	28-04-1994
		DE 68914110 T	07-07-1994
		EP 0372966 A	13-06-1990
		ES 2062041 T	16-12-1994
		IE 62927 B	08-03-1995
		JP 2218616 A	31-08-1990
EP 0341007 A	08-11-1989	AT 111360 T	15-09-1994
		CA 1339090 A	29-07-1997
		DE 68918155 D	20-10-1994
		DE 68918155 T	02-03-1995
		ES 2064439 T	01-02-1995
		JP 2071747 A	12-03-1990
		JP 2774141 B	09-07-1998
		US 5290552 A	01-03-1994

This Page Blank (uspto)

This Page Blank (uspto)